

Leadership in 2D living neural networks

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Abstract.

Eytan and Marom [1] recently showed that the spontaneous burst activity of rat neuron cultures includes ‘first to fire’ cells that consistently fire earlier than others. Here we analyze the behavior of these neurons in long term recordings of spontaneous activity of rat hippocampal and rat cortical neuron cultures from three different laboratories. We identify precursor events that may either subside (‘small events’) or can lead to a full-blown burst (‘pre-bursts’). We find that the activation in the pre-burst typically has a first neuron (‘leader’), followed by a localized response in its neighborhood. Locality is diminished in the bursts themselves. The long term dynamics of the leaders is relatively robust, evolving with a half-life of 23-34 hours. Stimulation of the culture can temporarily alter the leader distribution, but it returns to the previous distribution within about 1 hour. We show that the leaders carry information about the identity of the burst, as measured by the signature of the number of spikes per neuron in a burst. The number of spikes from leaders in the first few spikes of a precursor event is furthermore shown to be predictive with regard to the transition into a burst (pre-burst versus small event). We conclude that the leaders play a rôle in the development of the bursts, and conjecture that they are part of an underlying sub-network that is excited first and then act as nucleation centers for the burst.

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1. Introduction

Neurons that are extracted from the brain before they form connections can be grown in a dish, where they proceed to build a random network that is different from the one that they make within the brain [2, 3]. As this network matures it spontaneously develops an electrical activity that is characterized by collective *bursts* [4, 5, 6, 7]. The conceptual problems underlying spontaneous excitation of the network as a whole involve both the initiation and the propagation of the activity. Models of initiation usually rely on the existence of a constant level of random firing in the network, contributing to background noise that rises at times to a level that suffices to excite the network.

When the neurons are restricted to grow along lines they create 1D cultures and their firing pattern can be well understood in terms of a generation process that occurs in one of several Burst Initiation Zones (BIZ), and a subsequent propagation from that area into the rest of the line [8, 9]. In 2D the situation is much less clear. While numerous studies identify BIZ's in 2D as well, the course of the outward propagation is not concentric and is difficult to follow [10, 4, 11, 12]. Since signals are confined to propagate along the axons that are linear and not space filling, advance of the signal in the plane must be the collective effect of many neurons.

Eytan and Marom [1] recently found that in 2D a select group of neurons, termed 'first-to-fire', or 'precursor' neurons, begin to fire ahead of the rest of the network, and were seen to do so consistently for the several hours that they were monitored. This raises several interesting questions regarding their rôle in activating the network. Are these neurons simply better connected to the actual initiation zone, or do they actively participate in creating and propagating the burst. Since the experiments are performed with multi electrode arrays that typically monitor only about 0.1% of the neurons in the culture, a further question is, how many such 'first to fire' groups exist in the whole culture and how do they interact with each other. The possibility of a 'sub-network' of such neurons that ignites the rest of the culture is an intriguing one.

In this study we explore the collective mechanism for the initiation of the burst. Our approach will be to look first into the individual structure of each burst and to study the properties of the 'first-to-fire' neurons. We then ask whether and how their activity actually predicts properties of the burst that follows.

2. Methods

2.1. Neural Culture Types

We analyzed data from two-dimensional cultures prepared in three different protocols taken in three different labs. They are classified according to their respective feeding protocols, which we found to be the most significantly differentiating parameter of the growth and preparation conditions:

- 'CF' for a Continuously-Fed culture, prepared from cortices of post-natal rats that are

perfused [1], from the Marom lab.

- ‘*DF*’ for Daily Fed cultures, prepared from hippocampi of pre-natal E19 rats, from the Moses lab.
- ‘*WF*’ for cultures fed every 5-7 days, prepared from cortices of E18 rats [7], from the Potter lab.

The three culture types are compared in Table 1 below.

Name	Feeding Schedule	Source	Culture diameter [mm]	Feeding Medium	Array size, $X \times Y$ [mm] and D [μm]	no. of cultures
<i>CF</i> [1]	continuous perfusion from DIV 14	Rat cortical, postnatal day 1	15	5% horse serum	1.4×1.4 and 200	1
<i>DF</i>	33% medium replacement, daily	Rat hippocampal, prenatal day 19	15	10% horse serum	2.5×4.5 and 500	9
<i>WF</i> [7]	50% medium replacement, every 5 days	Rat cortical, prenatal day 18	4	10% horse serum	1.4×1.4 and 200	4

Table 1. Culture types used in this work. $X \times Y$ are the array dimensions, D is the inter-electrode distance, and DIV stands for days in-vitro.

2.2. Preparation and Measurement of *DF* Cultures

Here we only describe the preparation of *DF* cultures. The preparation of *CF* is described in [1], and the preparation of *WF* cultures in [7].

MEA Preparation. Multi-electrode arrays (MEA) of 6×10 electrodes with $500\mu\text{m}$ spacing were used (MultiChannelSystems, Reutlingen, Germany). The MEA were prepared using overnight application of PEI solution (Poly Ethylene Imine, Sigma, 0.05% in borate buffer). 2 hours prior to cell plating, the MEA was washed 4 times with double distilled water, and left with plating medium.

Culture Preparation and Maintenance. Rat hippocampal neurons were taken from embryos of 19 day pregnant Wistar rats. The dissection and plating were done according to [13, 14], with daily feeding of the culture. Briefly, the hippocampus was mechanically dissociated, and cells were plated in a concentration of 12000 cells/ mm^2 in 3 ml plating medium (5% fetal calf serum (FCS, Sigma) and 5% heat inactivated horse serum (HIHS, Sigma) in Eagle’s MEM (Sigma) enriched with 0.6% glucose, 2 mM glutamine, and 15mg/ml Gentamicin (MEM+3g),

enriched with B-27 supplement). In day in-vitro (DIV) 3–6 the culture was fed daily with 1ml of changing medium which is comprised of MEM+3g, 5% FCS, 5% HIHS with 20mg/ml FUDR and 50mg/ml Uridine (both from Sigma), and from then on it was fed with 1ml final medium which includes MEM+3g with 10% HIHS. The resulting neuron density is about 2000 cells/mm².

Culture Measurement. Measurements were performed at 37°C, in a dry 5% CO₂ incubator. To minimize evaporation of the growing medium, the culture chambers were covered with a thin teflon foil [15]. For each culture, we measured regularly and in a continuous way the spiking activity every 24 hours, in DIV 5–35. The measurements for each culture were performed over times ranging between 1 to 24 hours, from which extracts lasting 20 to 30 minutes were analyzed. Measurements which occurred less than 15 minutes after moving the culture or less than 6 hours since the last feeding were discarded. Special care was taken to assure minimization of water loss from the growing medium ([14, 16]).

Spike Detection. The detection was done after [14]. The output from the MEA1060 amplifier (MultiChannelSystems, Germany) was sampled at 20kHz (PCI-6071E, National Instruments, Austin, TX). The condition for spike detection was that the absolute value of the sampled signal exceeds the threshold for at least 0.2ms. The threshold was set at the maximum of 15 μV and 3 times the signal standard deviation (which is $\sim 3.5 \mu V$). Further spikes with opposite polarity and less than 3ms apart were disregarded as they were usually caused by overshoot. Spike shape discrimination was not performed, so that the analysis relies only on the spike timing and the electrode position. The number of neurons recorded from each electrode was estimated separately by spikes sorting as 1–4, with an average of 2.5.

Culture Stimulation. We used stimulation to modify the leader distribution, in two methods. The first method used one of the recording electrodes, to which a 0.6–1 Volts amplitude, 1ms bipolar pulse pair with 10ms inter-pulse interval was injected. The second method was bath stimulation, where two platinum electrodes were immersed in the growing medium 4mm aside from the electrode array center. The bath stimulation was not carried out directly over the electrode array to minimize the measurement dead time caused by the stimulation current. The electrodes were exposed for 10mm, spaced 2.5mm apart and were less than 1mm above the neuron level. In this case, bipolar pulses with an amplitude of 2–3.5 Volts and width of 20ms were used.

For both methods we used a battery powered current source which is isolated from the surrounding environment to minimize electrical noise in the multi-electrode measurement. The culture was monitored in order to verify its response to the stimulation. The voltage of the stimulation was adjusted to cause a culture-wide response in at least 50% of the stimulations when the stimulation frequency was set to the spontaneous bursting frequency.

2.3. Data Analysis

2.3.1. Time course Cultures were observed in days in-vitro 5–30 or parts within. Measurements were taken typically about once a day, on the order of an hour. Each daily data set is termed *epoch*. There were on average 8, 25 and 20 epochs in *CF*, *DF*, *WF*, respectively. We looked for changes of behavior across epochs, typically finding changes towards the middle of the measurement period. This naturally leads to a division of the epochs into two *superepochs*. Electrodes were included in the analysis of a given epoch (‘active electrode’) if they fired at least once in this epoch.

2.3.2. Burst definition The definition of bursts is based on the list of spikes which are obtained in the measurements. For each spike i , the list gives us t_i , the time of the i th spike, and c_i , the electrode (‘channel’) of that spike.

We will associate each spike with one of 4 classes: *in-burst*, *pre-burst*, *small-event*, *isolated*. Basically, a spike is in a burst if it is in a group of many spikes which follow each other closely. It is in a pre-burst or small-event if it is not in a burst and is in a sequence of spikes close enough in time so that communication between them is still possible. The distinction between pre-burst or small event depends on whether the spike is eventually followed by a burst or not. Finally, all other spikes are isolated. The reader may want to refer to Fig. 2 for the definitions which follow.

More precisely, we use three parameters, n_{burst} , δt_{burst} , and $\delta t_{\text{isolated}}$. We first define consecutive sets, separated by interspike gaps of length at least $\delta t_{\text{isolated}}$. Thus the set of spikes is divided into sets \mathcal{R}_ℓ of consecutive spikes, with the property that if i and $i+1$ are in \mathcal{R}_ℓ then $t_{i+1} - t_i \leq \delta t_{\text{isolated}}$, while for $i \in \mathcal{R}_\ell$ and $i+1 \in \mathcal{R}_{\ell+1}$ we have $t_{i+1} - t_i > \delta t_{\text{isolated}}$. The rationale is that the interspike gap is large enough so that no ‘memory’ remains between the spike at t_i and that at t_{i+1} when $t_{i+1} - t_i > \delta t_{\text{isolated}}$.

Each of the \mathcal{R}_ℓ contains at least one spike, but may contain many. We subdivide each \mathcal{R}_ℓ into 2 disjoint sets $\mathcal{R}_\ell = \mathcal{P}_\ell^{(0)} \cup \mathcal{B}_\ell^{(0)}$ using the following condition: We first define $i_* = i_*(\ell)$ to be the first index in \mathcal{R}_ℓ with the property: $t_{i_*+n_{\text{burst}}-1} - t_{i_*} < \delta t_{\text{burst}}$, that is, there are at least n_{burst} spikes in \mathcal{R}_ℓ within a lapse of time δt_{burst} . If the condition is never met, then the set \mathcal{R}_ℓ is not subdivided and is called a *small event* if it has more than 1 spike and is an *isolated spike* otherwise. For those cases where such an i_* can be found, we check if it is also the first spike in the burst. If so, then that burst is an *immediate burst* that has no *pre-burst*, and $\mathcal{R}_\ell = \mathcal{B}_\ell^{(0)}$. For all the other bursts (these are the majority) we divide $\mathcal{R}_\ell = \mathcal{P}_\ell^{(0)} \cup \mathcal{B}_\ell^{(0)}$ where $\mathcal{P}_\ell^{(0)}$ are the indices $i \in \mathcal{R}_\ell$ with $i < i_*$, and $\mathcal{B}_\ell^{(0)}$ are the others. The letters \mathcal{P} and \mathcal{B} refer to pre-burst and burst.

We now renumber the set of indices as follows

$$\{1, \dots, N\} = \mathcal{Q}_1 \cup \mathcal{P}_1 \cup \mathcal{B}_1 \cup \mathcal{Q}_2 \cup \mathcal{P}_2 \cup \mathcal{B}_2 \cup \dots,$$

where each of the \mathcal{P} and \mathcal{B} equals one of the earlier $\mathcal{P}^{(0)}$ and $\mathcal{B}^{(0)}$ but the \mathcal{Q} are empty sets or unions of consecutive \mathcal{R} ’s which were not identified as bursts in the earlier stage.

We call \mathcal{B}_ℓ the *burst* number ℓ , \mathcal{P}_ℓ the corresponding *pre-burst* and \mathcal{Q}_ℓ the corresponding *quiet phase*. The *leader* of burst ℓ is the channel c_{i_ℓ} , where i_ℓ is the number of the first spike in \mathcal{P}_ℓ .

Note that it may happen that \mathcal{Q}_ℓ does not contain any spike, but we always can define a quiet duration. This duration is the time between the last spike of $\mathcal{B}_{\ell-1}$ and the first spike of \mathcal{P}_ℓ . We define T as the combined length of the quiet phases. The length of the quiet phase \mathcal{Q}_ℓ is $t_{i_\ell} - t_{\hat{i}_{\ell-1}}$ ($> \delta t_{\text{isolated}}$) where i_ℓ is the first spike in \mathcal{P}_ℓ (i.e., the leader of burst ℓ) and $\hat{i}_{\ell-1}$ is the last spike of $\mathcal{B}_{\ell-1}$. Therefore,

$$T = \sum_{\ell} t_{i_\ell} - t_{\hat{i}_{\ell-1}},$$

is the total duration of all quiet phases.

The parameters which were used for the 3 cultures are shown in Table 2.

Culture Type	n_{burst}	δt_{burst} [ms]	$\delta t_{\text{isolated}}$ [ms]
<i>CF</i>	25	20	20
<i>DF</i>	10	40	50
<i>WF</i>	20	20	10

Table 2. Burst detection parameters.

With these definitions we can do now some statistics: We are interested in the activity of the system *outside* the bursts. The question is whether certain channels are the leaders of the neural activity more often than warranted by simple expectation.

So we start by estimating the expected rate (or spiking density) for each channel. Two choices can be used here: Averages over the \mathcal{Q}_ℓ or averages over \mathcal{Q}_ℓ and \mathcal{P}_ℓ . Let us continue with the first option, this is the one used in the experiments.

For each channel n , we define C_n as the number of times n has spiked in the set

$$\mathcal{Q} = \mathcal{Q}_1 \cup \mathcal{Q}_2 \cup \dots$$

The rationale for this is that some channels spike only in the bursts. So the channels that are able to be leaders are those which can spike in the quiet phase. However, we have checked that for most channels the activity in the quiet phase is comparable to that in the bursts (data not shown).

Leader Significance. For every pre-burst, small event or immediate burst, the electrode which fires first is called a *leader*. Since we are interested in their special rôle, we first need to make sure that leaders are not just electrodes with high activity, which therefore are statistically more often the first ones to fire. The following discussion will show that they are over-proportionally more often leaders.

Henceforth we only discuss bursts with a pre-burst and their *leaders*. We require that a leader's probability to lead bursts should be significantly higher than its probability to fire in

low-rate (quiet) intervals. Let M be the total number of bursts that have a pre-burst, which is also the total number of burst leaders. We consider a spike in the quiet interval, and term by q_n the probability that electrode n has fired that spike. The a-priori probability $P_n(k)$ that the channel n is k times the leader is then

$$\binom{M}{k} q_n^k (1 - q_n)^{M-k}.$$

In the limit of large M and reasonable q_n this distribution is approximated by a Gaussian of mean Mq_n and variance $Mq_n(1 - q_n)$. We denote by B_n the *actual* number of bursts with a pre-burst that electrode n leads (note that $\sum_n B_n = M$). Thus we have a scale on which to test leadership. We define α_n , a ‘leadership score’, and decide that an electrode is a *significant leader* if it is at least 3 standard deviations above the natural expectation value. This corresponds to a p-value of 0.001. The criterion for significant leadership is therefore

$$\alpha_n = \frac{B_n - Mq_n}{\sqrt{Mq_n(1 - q_n)}} > 3. \quad (1)$$

A second condition is aimed to eliminate electrodes that fire very seldom and lead a negligible number of bursts but are not screened by the first condition. In other words, an electrode cannot be a leader if the fraction of bursts it leads is less than a threshold of 3% of the bursts.

Mutual Information. We estimate the mutual information of two electrodes in a series of time intervals based on empirical probabilities, according to [17]. Using the division of time to pre-burst and burst intervals, we define a firing event in a given interval as $k = 0, 1$, where 0 stands for no spike and 1 stands for at least one spike. We did not use the quiet interval for this analysis. We denote by $N_n(k)$ the number of times electrode n had an event k in a series of N_{int} intervals. The probability assigned to event k is thus $p_n(k) = N_n(k)/N_{\text{int}}$. The number of joint events in which electrode n had event i and electrode n' had event j is given by $N_{n,n'}(i, j)$. The joint probability of events i, j for electrodes n, n' is then given by $p_{n,n'}(i, j) = N_{n,n'}(i, j)/N_{\text{int}}$. The mutual information between two electrodes is then given by

$$I_{n,n'} = \sum_{i,j=0,1} p_{n,n'}(i, j) \cdot \log \frac{p_{n,n'}(i, j)}{p_n(i)p_{n'}(j)}$$

3. Results

3.1. Neural Activity

All three culture types showed spontaneous bursting in most of the active electrodes. In the cultures from all sources *CF* (a single culture), *DF* (n=9 cultures), *WF* (n=5 cultures) the average rate of spikes per electrode per unit time gradually increased along the measurement period as did the bursting rate. The measurement period was (in DIV) 14–25 (*CF*), 5–30 (*DF*), and 5–30 (*WF*). The electrode firing rate (in Hz per active electrode) was 3–10 (*CF*), 1–3 (*DF*) and 4–10 (*WF*). For all cultures more than 85% of the intact electrodes were firing together in bursts after DIV 14.

The main difference between the activity of the cultures lies in the rate of firing. In particular, maximal bursting rates were (in Hz) 1.6 (*CF*), 0.3–1.4, with an average of 0.65 (*DF*), and 0.07–0.84, with an average of 0.26 (*WF*). These maximal rates were obtained in ages of DIV 19–24 (*CF*), 14–21 (*DF*), and 21–28 (*WF*). The difference between the bursting rates can mainly be attributed to the feeding differences - we have seen that more frequent feeding increases the bursting rate [14]. By the third week-in-vitro of the culture, about 90% of the spikes were within bursts for all three culture types.

WF cultures were special in that they contained fewer bursts but were more active in the quiet intervals between the bursts, with an average firing rate (excluding bursts) of about 0.1 Hz per electrode as compared to 0.002 Hz (*DF*) and 0.02 Hz (*CF*).

3.2. Existence of Leaders

Most of the bursts originated in a pre-burst (94% for *CF*, 86% for *DF*, and 73% for *WF* cultures).

The pre-burst lasted on average 230ms in *DF*, 180ms for *CF* and 22ms for *WF*. The average number of participating electrodes was 10 in *DF*, 18 in *CF* and 6 in *WF*. The *bursts* lasted on average 500ms in *DF*, 314ms for *CF* and 228ms for *WF*. The corresponding average number of firing electrodes was 29 in *DF*, 48 in *CF* and 31 in *WF*.

In all epochs, only 4 ± 1.9 (mean \pm SD), 2.9 ± 1.2 , and 2.0 ± 1.4 electrodes are significant leaders for more than 24 hours in *CF*, *DF* and *WF* cultures, respectively.

3.3. Time evolution

We are interested in finding out whether leadership is maintained over successive epochs. It turns out, that for all cultures, the most significant leaders are indeed stable over multiple epochs (Fig. 3). To quantify the leadership inertia, we define L_n as the set of epochs in which electrode n is a significant leader, *i.e.*,

$$L_n = \{k \mid \alpha_c < \alpha_{n,k}\}, \quad (2)$$

where $\alpha_c = 3$ is the threshold leadership score, and $\alpha_{n,k}$ is the leader score for electrode n in epoch k . For every consecutive sequence $k, k+1, \dots, \ell$ of integers in L_n , we define the lifetime of leadership by $t_{\ell+1} - t_k$.

The *distribution* of the lengths of all these time intervals follows an exponential distribution with half-life (in hours) of 23.1 ± 3.0 (mean \pm SE) (*CF*), 34.4 ± 4.4 (*DF*), and 29.5 ± 3.6 (*WF*).

The difference between the culture types is probably caused by the culture maintenance protocol.

3.4. Locality of activity

We assess the locality of electrode co-activation within the same burst or pre-burst, by measuring the Mutual Information as a function of distance. We compare the pre-burst to

the burst, and show that activity in the pre-burst is localized while the activity in the burst tends to spread all over the culture. We quantify the tendency to fire together by estimating the mutual information between electrode pairs (see Sect. 2). A pair of electrodes has higher mutual information if they participate in the same pre-burst or burst interval. We plot the mutual information $I_{n,n'}$ as a function of $d_{n,n'}$, the distance between the electrodes n and n' , averaging over all electrode pairs which have the same distance. The mutual information decreases with the inter-electrode distance for all interval types. In the pre-burst, we get a reasonable exponential fit of the form $I(d) = A \cdot \exp(-d/L)$ (for example, Fig. 4).

The mutual information displayed in Fig. 4 is normalized so that a direct comparison can be made between the burst and the pre-burst. However, the actual values of the measured information are also of interest. The mutual information in the burst is typically 5 times higher than in the pre-burst. This shows that in the burst, the co-activation is much higher than in the pre-burst. Looking at Fig. 4 one can see that in the pre-burst the mutual information decays gradually, going to zero at long distances. In contrast, during the burst it has a short initial decay, on the order of 1–2mm, beyond which the information is maintained at a constant value. We conclude that in the pre-burst the activity is primarily local, decaying to zero within 5mm. In contrast, in the burst the local effect is limited (within 1–2mm), and once the burst develops and advances outside this local region then all the culture behaves as one correlated region.

The value of L in the pre-burst interval can be measured reliably in epochs of maximal activity, occurring in DIV 19–23 (*CF*), 10–20 (*DF*) and 15–24 (*WF*). At other times, the decay is too small to calculate L reliably. In these days, L (in mm) of the pre-burst interval either wanders between 0.64–1.8 as function of culture age (*CF*), or takes constant values of 1.85 ± 0.16 (*DF*), 1.09 ± 0.02 (*WF*). In all epochs of maximal activity defined above, the localization in the burst interval as well as in the quiet phase is weaker than in the pre-burst interval.

As it is known that the recruitment of neural activity in bursts is based on synaptic transmission [2, 5], we relate the correlation length to the number of synaptic connections that the signal must propagate through. This in turn is determined by the axonal length, which is also in the range of about 1mm in similar cultures [18, 19].

3.5. Effect of stimulation

We have seen that the leaders can remain stable over long times when the culture is spontaneously active. We wished to assess whether the leaders can be changed using an external driving force [20]. In order to modify the leader distribution we stimulated the cultures using either one of the MEA electrodes or bath electrodes (see Sect. 2). These experiments were only done on two *DF* cultures, additional to the 9 cultures discussed above. The stimulation was repeated 40–240 times, in stimulation frequencies of 0.2–1 Hz during periods of 2–5 minutes for bath stimulation, and 500–1000 times in frequencies of 0.1–0.3 Hz during periods of 20–30 minutes for single-electrode stimulation. The response of the culture to the stimulation did not exceed the spontaneous bursting rate: stimulation in higher frequencies resulted in a lower fraction of bursts that did occur as a response to stimulation.

We assess the leader distribution measurement resolution by considering a combined all-channel criterion for measuring the modification in the leader distribution that is based on B_n , the number of bursts lead by electrode n . We define a similarity measure between two leader distributions $\mathbf{B}' = \{B'_n\}$ and $\mathbf{B} = \{B_n\}$ as their normalized scalar product,

$$S = \frac{\mathbf{B}' \cdot \mathbf{B}}{|\mathbf{B}'| \cdot |\mathbf{B}|} . \quad (3)$$

The similarity S is bounded between 0 and 1, where $S = 1$ means identical leader distributions while a similarity of 0 means that all the leaders have been completely replaced by new leader electrodes. The reference distribution B'_n was measured during a 30–60-minute interval which ends 15 minutes before the stimulation, and for B_n we used a sliding 10-minute interval either before ($t < 0$) or after ($t > 0$) the stimulation. Without stimulation, S slowly wanders due to distribution's dynamics, starting close to 1 and approaching values of 0.6–0.9 after 2 hours. To allow a clear comparison, we normalize the value of S by S_{ref} , its mean value during the 15 minutes prior to the stimulation.

Bath stimulation results are presented in Fig. 5 for cultures in age of 12–14 DIV. After the stimulation, the deviation rises abruptly, meaning that the short-term stimulation effect is maximal. The variation between experiments causes the large spread of results which is visible in short time after the stimulation. After 20–30 minutes, the similarity climbs back towards 1. In younger cultures aging 6–8 DIV, the average decline in similarity is three times higher, the variation between experiments is also three times higher, and the return to 1 somewhat faster (data not shown). The climb of similarity towards 1 indicates that the impact of the stimulation is fading. We note that opposite to the bath stimulation case, single electrode stimulation did not result in any modification in the leader distribution (results not shown).

We conclude that the bath stimulation effects are limited to less than an hour, and that the leader distributions of more mature cultures show lower susceptibility to stimulation.

3.6. Predictability of activity

The leader electrodes can be, on one hand, sensitive probes of the average whole-culture activity, or, on the other hand, be the areas that are leading the recruitment of the rest of the culture. In order to judge between these alternatives, we have been able to identify properties of the burst that correlate with the identity of the leader. In the following paragraphs, we show that the identity of the leader affects the activity following it in both the pre-burst and the burst intervals.

To test the relation between the leaders and the bursts, we analyzed the data in two ways. First, we determine a small number n of leaders, and for every burst initiated by that leader, we determine the number of spikes in each channel. Thus, we have a sequence of vectors $\mathbf{c}_{\ell,i}$, where the j 'th element of the vector $\mathbf{c}_{\ell,i}$, is the number of spikes in channel j for the i^{th} burst that had a leader ℓ . We take the average of these vectors, normalized to norm 1:

$$\mathbf{c}_{\ell} = \sum_i \mathbf{c}_{\ell,i} / N_{\ell} ,$$

where N_ℓ is the number of bursts with the leader ℓ . Investigation of these vectors shows that they are practically indistinguishable to the eye.

However, a prediction can be made for every burst whose leader is one of the ℓ above, as follows: If \mathbf{C} is the (normalized) vector corresponding to the spike count in one specific measured burst, we form the scalar products $\mathbf{C} \cdot \mathbf{c}_\ell$ and predict that the leader is that ℓ for which this quantity is largest. We do this for every burst. But we also know who was actually the leader of the burst \mathbf{C} , say ℓ' and we therefore get a matrix $A_{\ell',\ell}$ of counts of predictions ℓ for the case when ℓ' was the actual leader. In a perfect network, the matrix A would be diagonal, since the prediction would never be wrong. This is too much to expect for the noisy and untrained neural network we are studying, but the data show clearly a high correlation between the predicted and the true leader. In a totally random situation, the result would be evenly distributed between the n leaders and the relative abundance would be $A_{\ell',\ell} / \sum_\ell A_{\ell',\ell} = 1/n$. Thus we show in Table 3 the quantities $nA_{\ell',\ell} / \sum_\ell A_{\ell',\ell}$, and a coefficient > 1 indicates a positive correlation of the prediction.

	22	32	44	55	57		12	19	40	60
22	2.43	0.19	1.08	0.87	0.43	12	3.28	0.29	0.20	0.24
32	1.24	0.99	1.59	0.73	0.45	19	0.67	2.40	0.61	0.32
44	1.41	0.47	2.29	0.50	0.33	40	0.86	0.16	2.40	0.59
55	1.18	0.60	0.71	1.78	0.74	60	1.80	0.26	1.21	0.73
57	0.78	0.30	0.54	0.89	2.49					

Table 3. Two examples of the correlations between the leader (left column) and the type of burst (top row). The left matrix is for superepoch 2 of the *CF* culture and the right is for superepoch 2 of a *DF* culture. Note the much higher scores on the diagonal (values higher than the average by more than one standard deviation are colored red), as well as a small uncertainty for the pair of close-by electrodes (22 and 32) in the left matrix.

A second test for the relation of leaders and bursts is done by another prediction we make regarding the activity that follows a slow initiation. This activity may increase to become a burst, or die away as a small event. Our prediction is based on the number of leader electrodes that participate in the first spikes. It turns out that when more leaders are active in the first few spikes, the ratio between the probability of the activity to become a burst to the probability to become a small event increases significantly (Fig. 6).

4. Discussion

The presence of leaders is an interesting fact, and we now analyze some of the consequences and problems related to it. We have seen that the leaders are first of all localized and stable, to which we now add the assumption that they are homogeneously dispersed throughout the whole culture, although we have not measured this. This assumption seems very reasonable, since leaders are observed in all three preparations, and the location of the electrodes is

not expected to be special in any way. Although the electrodes cover an area that is only a small part of the culture (typically reporting on less than 0.1% of the neurons), they almost invariably succeed in picking up some leaders.

Two observations make us conjecture that ‘leaders’ not only report on the burst, but are active in causing it. The first is the locality of activity in the pre-burst, indicating a recruitment process, [12], in other words transfer of information from the leader to its surrounding neurons prior to the transition of the whole network to a full burst. This is reminiscent of a similar recruitment process around BIZ’s in 1D networks, in which transition from low amplitude, localized activity to higher amplitude, fast propagating activity was observed [8], and that is related to the buildup of synchrony in neural firing [14]. If the bursts were coming from outside the monitored area then we would expect them to appear abruptly, with no pre-burst and no gradual growth like the one we observe. So, although we only observe a small part of the culture, leaders are clearly identified in the field of observation for most of the bursts, indicating that the burst generation must have a local component in its genesis.

The second observation is the predictive power of the leaders as to the ‘character’ of the burst. Several groups have reported on the existence of a limited number of firing patterns in the bursts [5, 10, 21]. We have seen additionally that the spiking pattern of each electrode within the burst provides a signature that suffices to statistically identify its leader. Similarly, the firing pattern of leaders in the first few spikes of the small event or the pre-burst allows a prediction as to whether a burst will form or not.

We conclude from this that a relationship must exist between a burst and local leaders, emphasizing again that this is by no means just a statistical abundance of spiking by the leaders, but a sign of signaling between the leader, the exterior parts of the sample, and the burst generation.

What then do these leaders have as input that makes them fire first? The dominant signal inputting on a given neuron is usually thought to arise from the firing of other neurons rather than from environmental cues such as fluctuations in the amount of ions in the surrounding fluid. The reason that the leaders are first to fire may be that they have the most inputs, or that they are more sensitive to excitation. Recall however, that they do not fire particularly often.

There are three scenarios for how the leaders are excited. In the simplest case one leader in the whole culture is excited by a localized low-level background activity. By becoming very active it will recruit first its nearby environment and then gradually the rest of the network. This scenario is rejected within our picture: we have argued that several leaders are being excited concurrently, otherwise we would not have seen leaders in the small region in which we measure. The second scenario involves a number of leaders being excited simultaneously by a widespread low-level noise that can activate many of the leaders in parallel. In this case they each excite a region around them, and these coalesce at random as the whole network bursts. The argument against this scenario is that we would not expect then a variability in the signature of the burst, since all bursts would evolve essentially in the same route, with the leaders being excited first and then recruiting their respective neighborhoods.

The third, most tentative yet most attractive scenario posits the existence of an underlying ‘sub-network’ connecting potential leaders. Their activation is triggered by the noise, but

here a small amount of background firing can suffice to set off the burst since the leaders first communicate amongst themselves and only then initiate the activity of other leaders. Variations in the noise that initially sets off the network could then cause variation in the response of the leader sub-network, effecting the parts of it that are recruited. This in turn reflects on the details and signature of the burst. This scenario is particularly intriguing since it implies a stochastic percolation behavior in the sub-network of the leaders which, once they have fired, act as nucleation sites for the whole network. Perhaps one could answer the questions raised in this discussion experimentally, first measuring passively and identifying leaders and then intervening by selectively suppressing the activity of the neurons near those electrodes with leader properties.

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Bibliography

- [1] D. Eytan and S. Marom. Dynamics and effective topology underlying synchronization in networks of cortical neurons. *J Neurosci*, 26(33):8465–76, 2006.
- [2] S. Marom and G. Shahaf. Development, learning and memory in large random networks of cortical neurons: lessons beyond anatomy. *Q Rev Biophys*, 35(1):63–87, 2002.
- [3] J.-P. Eckmann, O. Feinerman, L. Gruendlinger, E. Moses, J. Soriano, and T. Tlusty. The physics of living neural networks. *Phys Rep*, 449:54–76, 2007.
- [4] A. Tscherter, M.O. Heuschkel, P. Renaud, and J. Streit. Spatiotemporal characterization of rhythmic activity in rat spinal cord slice cultures. *Eur J Neurosci*, 14(2):179–190, 2001.
- [5] E. Maeda, H.P. Robinson, and A. Kawana. The mechanisms of generation and propagation of synchronized bursting in developing networks of cortical neurons. *J Neurosci*, 15(10):6834–6845, 1995.
- [6] M.H. Droge, G.W. Gross, M.H. Hightower, and L.E. Czisny. Multielectrode analysis of coordinated, multisite, rhythmic bursting in cultured CNS monolayer networks. *J Neurosci*, 6(6):1583–1592, 1986.
- [7] D.A. Wagenaar, J. Pine, and S.M. Potter. An extremely rich repertoire of bursting patterns during the development of cortical cultures. *BMC Neurosci*, 7(11), 2006.
- [8] O. Feinerman, M. Segal, and E. Moses. Signal propagation along unidimensional neuronal networks. *J Neurophysiol*, 94(5):3406–3416, 2005.
- [9] O. Feinerman, M. Segal, and E. Moses. Identification and dynamics of spontaneous burst initiation zones in uni-dimensional neuronal cultures. *J Neurophysiol*, 97(4):2937–2948, 2007.
- [10] J. van Pelt, I. Vajda, P.S. Wolters, M.A. Corner, and G.J. Ramakers. Dynamics and plasticity in developing neuronal networks in vitro. *Prog Brain Res*, 147:173–188, 2005.
- [11] P. Darbon, L. Scicluna, A. Tscherter, and J. Streit. Mechanisms controlling bursting activity induced by disinhibition in spinal cord networks. *Eur J Neurosci*, 15(4):671–683, 2002.
- [12] C. Yvon, C. Rubli, and J. Streit. Patterns of spontaneous activity in unstructured and minimally structured spinal networks in culture. *Exp Brain Res*, 165(2):139–151, 2005.

- [13] M. Papa and M. Segal. Morphological plasticity in dendritic spines of cultured hippocampal neurons. *Neurosci*, 71(4):1005–1011, 1996.
- [14] S. Jacobi and E. Moses. Variability and corresponding amplitude-velocity relation of activity propagating in one-dimensional neural cultures. *J Neurophysiol*, 97(5):3597–3606, 2007.
- [15] S.M. Potter and T.B. DeMarse. A new approach to neural cell culture for long-term studies. *J Neurosci Meth*, 110(1-2):17–24, 2001.
- [16] S.M. Potter. Distributed processing in cultured neuronal networks. *Progress in Brain Research*, 130:49–62, 2001.
- [17] J.P. Eckmann, E. Moses, and D. Sergi. Entropy of dialogues creates coherent structures in e-mail traffic. *PNAS*, 101(40):14333–14337, 2004.
- [18] W.P. Bartlett and G.A. Banker. An electron microscopic study of the development of axons and dendrites by hippocampal neurons in culture. I. Cells which develop without intercellular contacts. *J Neurosci*, 4(8):1944–1953, 1984.
- [19] A.R. Kriegstein and M.A. Dichter. Morphological classification of rat cortical neurons in cell culture. *J Neurosci*, 3(8):1634–1647, 1983.
- [20] E. Maeda, Y. Kuroda, H. P.C. Robinson, and A. Kawana. Modification of parallel activity elicited by propagating bursts in developing networks of rat cortical neurons. *Eur J Neurosci*, 10(2):488–496, 1998.
- [21] R. Segev, I. Baruchi, E. Hulata, and E. Ben-Jacob. Hidden neuronal correlations in cultured networks. *Phys Rev Lett*, 92:118102, 2004.

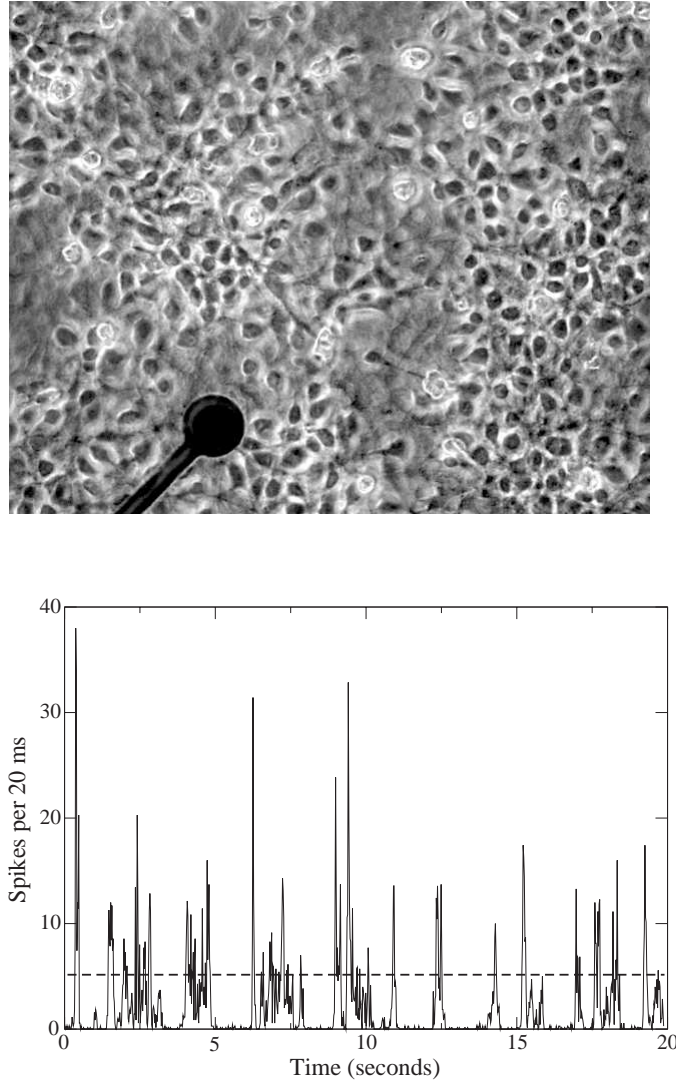
Figures and captions

Figure 1. a. Two-dimensional rat hippocampal neuron culture (*DF*) growing near one of the 60 measuring electrodes of the MEA. Electrode diameter is $30\mu m$. Phase micrograph taken using Zeiss Axiovert 135TV. b. Example of spontaneous bursting activity from a *DF* culture. The number of spikes in 20ms bins is drawn as a function of time. Burst detection threshold is 10 spikes in 40ms (dashed line).

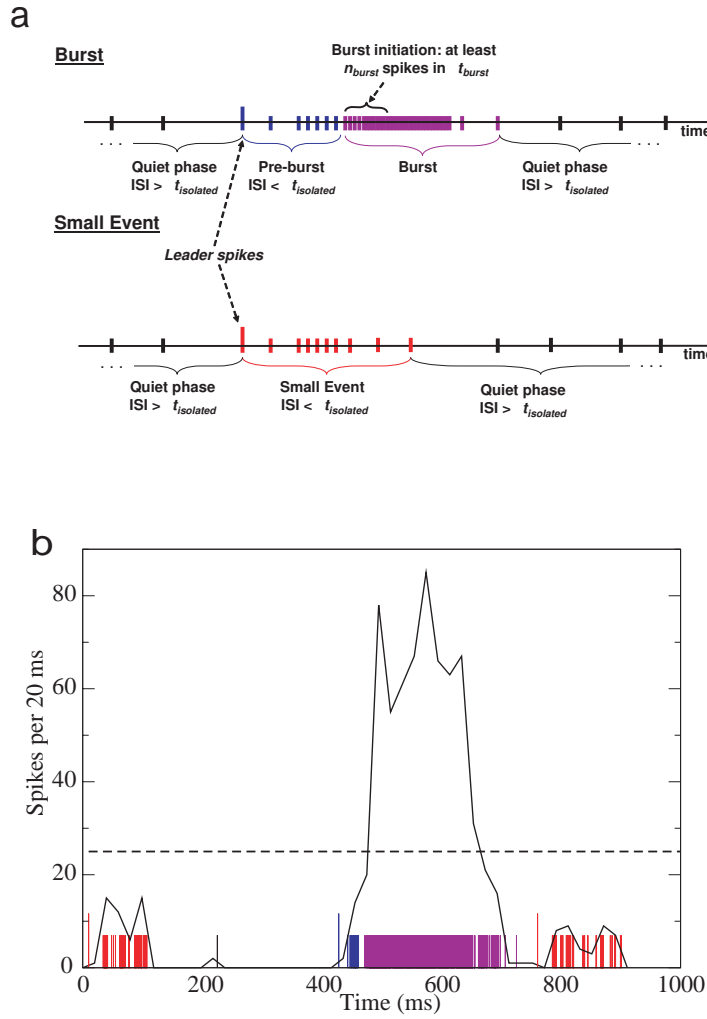


Figure 2. Burst identification. a. A Schematic drawing of the classification of spikes. The vertical lines indicate spikes, with the following color-code: Violet (burst), blue (pre-burst), red (small event), black (isolated events). Spikes with inter spike interval below $\delta t_{isolated}$ are grouped into a pre-burst that is followed by a burst if the spike density exceeds n_{burst} spikes in δt_{burst} (top timeline), or into a small event otherwise (bottom timeline). b. Example of spontaneous bursting activity in a CF culture. Single spikes are depicted as vertical lines with the color-code as in a, while the number of spikes per 20ms bins are drawn as a line. This data set includes one *isolated spike*, two *small events* and one *burst*. The leader spikes are shown with a somewhat longer vertical line. The dashed line is the threshold value $n_{burst} = 25$ in $\delta t_{burst} = 20\text{ms}$ for CF.

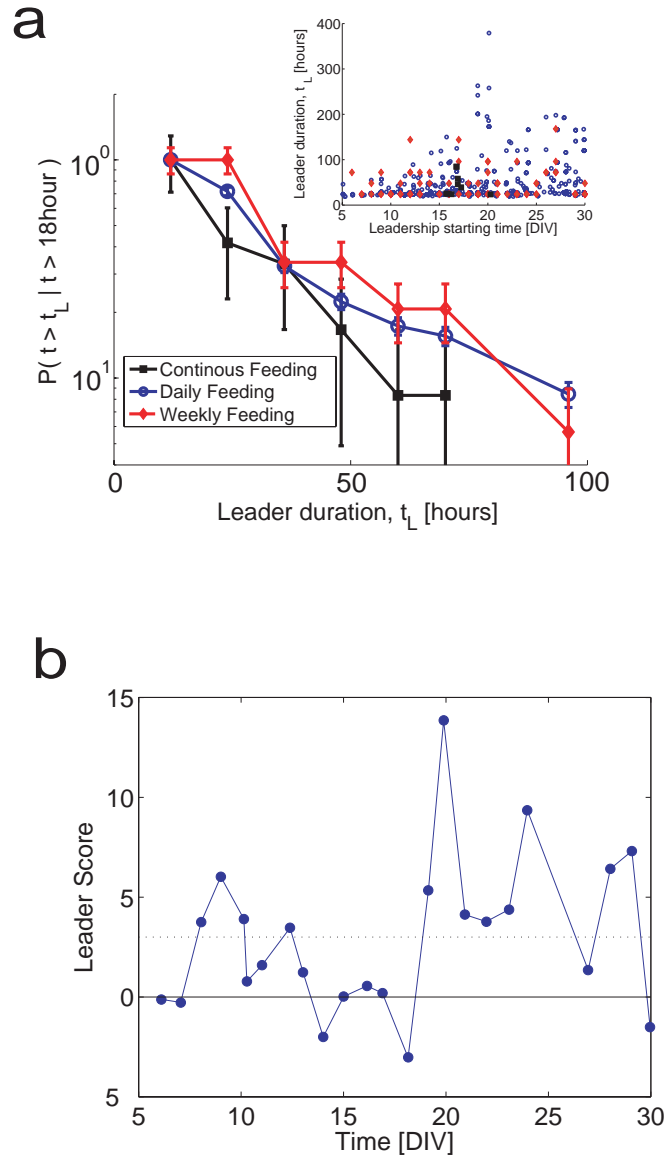


Figure 3. Leader electrodes remain with a high leadership score for days. a. The distribution of the leadership duration, for the three culture types, *CF*, with continuous feeding (black squares), *DF*, with daily feeding (blue circles), *WF*, with semiweekly feeding (red diamonds). Inset: leadership time durations of all leaders remaining at least 18 hours, as a function of the leadership starting time (marker code as in a.). The b. Leadership score as a function of day *in-vitro*, for an example leader electrode of *DF*.

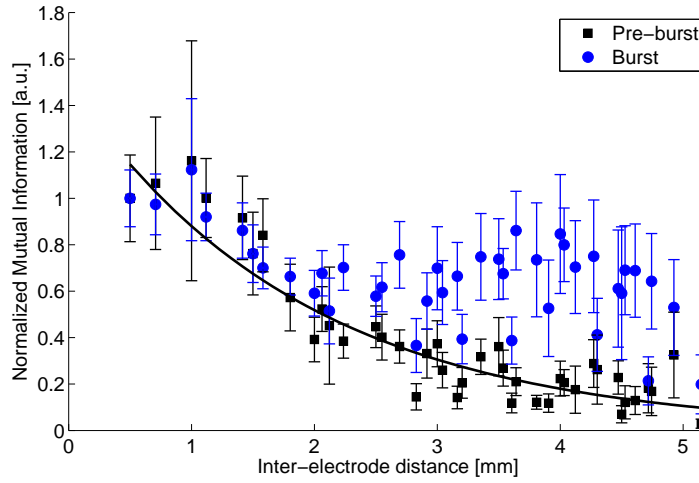


Figure 4. Average Mutual Information (MI) as a function of distance for pre-burst and burst intervals. The MI is normalized by its value at 0.5mm. The activity landscape is more localized in the pre-burst, and can be fit to an exponential (black line) with length scale of 1.3mm. The length scale in the burst is higher (blue line is a guide to the eye).

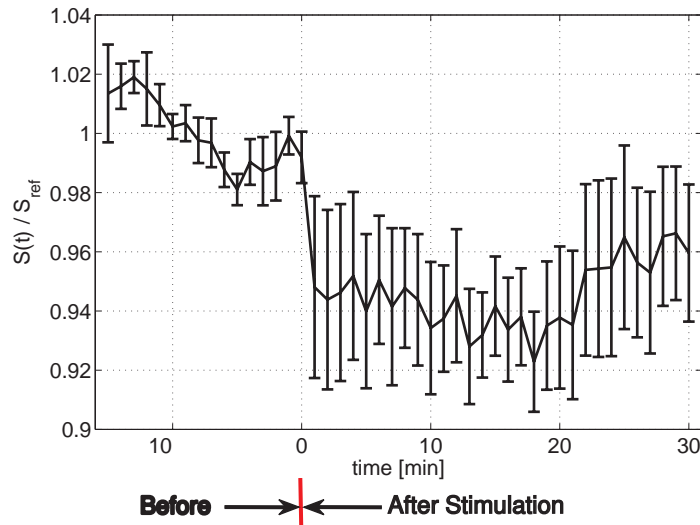


Figure 5. Stimulation drives changes in Leader distribution, that fade out within few tens of minutes. $S(t)$ is the similarity between the leader distributions of 30–60min pre-stimulation, and a sliding 10min window either pre-stimulation ($t < 0$) or post-stimulation ($t > 0$). The value of S is normalized by its average during the 15 minutes prior to stimulation. Vertical red line border between pre- and post-stimulation regions: note that the stimulation period, of 2–10min, is not present in the graph. $S(t)$ declines immediately following the burst and climbs up after about 30min.

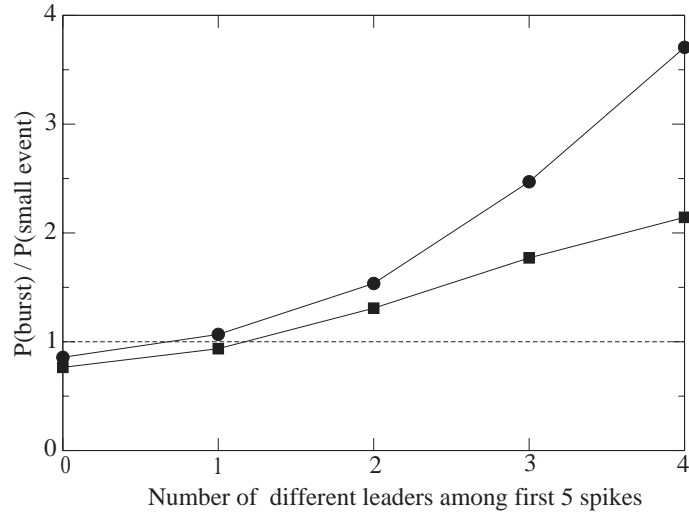


Figure 6. Prediction of burst initiation probability from the occurrence of leaders in the first spikes in the pre-burst or small event. The ratio of probabilities between burst and small event increases as a function of the number of leaders in the 5 first spikes in the pre-burst. Results are given for superepoch 1 (squares) and superepoch 2 (circles) of *CF*.